NANOCAPSULE ENCAPSULATION SYSTEM AND METHOD

CROSS-REFERENCE TO RELATED APPLICATION(S)

This application is a continuation of Application No. 09/796,575 filed February 28, 2001, which claims the benefit of U.S. Provisional Application No. 60/185,282 filed February 28, 2000.

BACKGROUND OF THE INVENTION

The present invention generally relates to a field of controlled-release delivery systems for macromolecules, particularly those for nucleic acids and gene therapy. More specifically, the present invention relates to nanocapsules having a diameter of less than about 50 nanometers, in which a bioactive component is located in a core of the nanocapsule, and to methods of forming these nanocapsules.

Over the past several decades, active and extensive research into the use of nanoparticles in the delivery of bioactive agents has generated a number of approaches in the preparation of nanoparticles. These approaches typically include the use of heat, high pressure homogenization, or high intensity ultrasound sonication to prepare nanoparticles having a diameter of more than 100 nanometers, or high amounts of solvents or oils, cytotoxic chemicals, such as cross-linking agents, adjuvants, catalysts or any combination of any of these, to prepare nanoparticles having a diameter of less than 100 nanometers. Furthermore, these approaches are challenging due to a number of variables.

For example, when organic solvents are included in the manufacturing process for nanoparticles, the organic solvent may denature the bioactive agent which reduces most, if not all, efficacy of the bioactive agent. In fact, denaturation of the bioactive agent may promote a toxic response upon administration of the nanoparticle, to a human subject, for example.

In addition, when an organic solvent is used to prepare nanoparticles, the organic solvent or solvent soluble polymer may undergo degradation to form a low pH environment that destroys the efficacy of the bioactive agent. Therefore, organic solvents may generally denature the bioactive agent during *or* after preparation of a nanoparticle.

As a result, organic solvents are typically removed during the manufacturing process of nanoparticles. However, inclusion of one or more organic solvent removal techniques generally increases the costs and complexity of forming nanoparticles.

The incorporation of high pressure homogenization or high intensity ultrasound sonication to prepare nanoparticles typically results in entangling or embedding the bioactive agent in a polymeric matrix of the nanoparticle. Entangling or

embedding the bioactive agent in the polymeric matrix may also denature the bioactive agent to thereby reduce the efficacy of the bioactive agent.

Entangling or embedding the bioactive agent in the polymeric matrix of the nanoparticle may also reduce the efficacy of the bioactive agent by permitting premature release of the bioactive agent *prior* to reaching a target cell. Premature release of the bioactive agent typically promotes cytotoxicity or cell death during administration of the nanoparticle.

Furthermore, nanoparticles that reach the target cell are typically transported into the target cell via endosomal regulated pathways that results in lysosomal degradation of the bioactive agent *and* the nanoparticle. Therefore, functional activity of the bioactive agent inside the target cell may not occur since the bioactive agent and the nanoparticle undergoes degradation. As used herein, the term "functional activity" refers to an ability of a bioactive agent to function within a target cell for purposes of providing a therapeutic effect on the target cell.

Additionally, high pressure homogenization or high intensity ultrasound sonication techniques often require complex and expensive equipment that generally increases costs in preparing nanoparticles. Therefore, an urgent need exists to prepare nanoparticles without the use of cytotoxic chemicals like organic solvents or the use of complex and expensive equipment. Furthermore, an urgent need exists to prepare nanoparticles that do not entangle nor embed the bioactive agent in the nanoparticle so that cytotoxic responses are minimized. Additionally, an urgent need exists to develop a nanoparticle that may be transported into a target cell where the bioactive agent is released to accomplish therapeutic delivery of the bioactive agent.

BRIEF SUMMARY OF THE INVENTION

The present invention generally relates to nanocapsules and methods of preparing these nanocapsules. The present invention includes a method of forming a surfactant micelle and dispersing the surfactant micelle into an aqueous composition having a hydrophilic polymer to form a stabilized dispersion of surfactant micelles. The method further includes mechanically forming droplets of the stabilized dispersion of surfactant micelles, precipitating the hydrophilic polymer to form precipitated nanocapsules, incubating the nanocapsules to reduce a diameter of the nanocapsules, and filtering or centrifuging the nanocapsules.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic of a method of the present invention for preparing nanocapsules.

FIG. 2A: "Nanocapsules prepared under different dispersion conditions" illustrates atomic force microscopy of nanocapsule formulations prepared under different dispersion conditions.

FIG. 2B: "Cumulative release studies for nanocapsule formulations" illustrates results from an experiment documenting quantitative recovery of small amounts of DNA from releasing solutions.

FIG. 2C: "Quantitative recovery of DNA from receiver solutions" illustrates cumulative release over 72 hours for nanocapsules prepared under different dispersion conditions.

FIG. 3: "Nanocapsule modulation of cellular uptake" illustrates relative pinocytotic activity of HacaT keratinocyte cultures treated with DNA complexes, nanocapsules containing DNA or no treatment.

FIG. 4: "Dose response for a nanocapsule formula" illustrates western blotting of total protein from rat fibroblast cultures.

FIG. 5A: "Nanocapsule-delivered transgene production in porcine dermis" illustrates western blotting of porcine dermal tissue.

FIG. 5B: "Macromolecule delivery across keratinized barrier epithelial" illustrates immunofluorescence microscopy of porcine dermal tissue sections from organ culture study demonstrating topical nanocapsule delivery across keratinized barrier epithelial.

FIG. 6: "Incorporation of nanocapsules into a suture" shows incorporation of nanocapsules into a solid dosage form.

FIG. 7A: "PVP nanocapsules are taken up by fibroblasts but not keratinocytes" illustrates polyvinylpyrolidone nanocapsule uptake and Green Fluorescent Protein (GFP) expression in 35 mm human dermal fibroblast and immortalized keratinocyte cultures.

FIG. 7B: "Nanocapsule design for tumor-targeting" illustrates tumor targeting of GFP plasmid DNA by Tenascin nanocapsules.

FIG. 7C: "Nanocapsule coating design for increased drug safety" illustrates an effect of nanocapsules that are coated with Tenascin and nanocapsules that are not coated with Tenascin on growth inhibition of squamous cell carcinoma and human dermal fibroblast (HDF) cultures.

FIG. 8A: "Cellular uptake and lysosomal sequestration of RNA oligimers complexed with polyethyleneimine" shows uptake of HDF cultures treated with nanocapsules containing 20 mer Fitc-labeled O-methyl RNA oligonucleotides.

FIG. 8B: "Nanocapsules avoid lysosomal sequestration at 18 hours post-addition" shows uptake of HDF cultures treated with nanocapsules containing 20 mer Fitc-labeled O-methyl RNA oligonucleotides.

DETAILED DESCRIPTION

The present invention generally relates to nanocapsules having a diameter of less than about 50 nanometers (nm). The present invention also relates to a method of preparing these nanocapsules. According to the method of the present invention, a nanocapsule is formed by partitioning a bioactive component within a core of surfactant molecules, and surrounding the surfactant molecules with a biocompatible polymer shell.

A method for producing the nanocapsule is generally depicted at 10 in Figure 1. In the method 10, a bioactive component 12 is homogeneously dispersed into a first aqueous composition 14 to form a hydrophilic composition (not shown). Next, a surfactant composition 16, including a surfactant component (not shown) that contains a plurality of surfactant molecules, and an optional biocompatible oil component 18, are introduced into a first dispersing apparatus 20 along with the hydrophilic composition. The surfactant composition 16 is subjected to conditions in the first dispersing apparatus 20 that initiate at least partial adsorption of the surfactant molecules onto a surface of the bioactive component 12.

Partial adsorption of surfactant molecules onto the surface of the bioactive component 12 initiates partitioning of the bioactive component 12 into a core of a shell formed from the surfactant molecules in the first aqueous composition 14. Adsorption of the surfactant molecules onto the surface of the bioactive component 12 may proceed until an entire surface of the bioactive component 12 is covered by the surfactant molecules to complete partitioning of the bioactive component 12 into the core of surfactant molecules and form a surfactant micelle 22.

Next, a biocompatible polymer component 24 is added to the surfactant micelle 22 to stabilize the surfactant micelle 22 located in the first aqueous composition 14. Preferably, the biocompatible polymer component 24 surrounds the surfactant micelle 22 in a stabilizing apparatus 26 to form a stabilized surfactant micelle 28.

After stabilization, the stabilized surfactant micelle 28 is transferred from the stabilizing apparatus 26 into a second aqueous composition 30 located in a second dispersing apparatus 32. Preferably, the second aqueous composition 30 includes a solute (not shown) that is capable of precipitating the biocompatible polymer component 24 that

coats the stabilized surfactant micelle 28. After precipitating the biocompatible polymer component 24 of the stabilized surfactant micelle 28, dispersed, optionally atomized precipitated nanocapsules 36, hereinafter referred to as nanocapsules 36, are formed.

It has been discovered that dispersing a surfactant composition, that includes a surfactant component having a hydrophile-lipophile-balance (HLB) value of less than about 6.0 units, into an aqueous composition that contains a bioactive component forms surfactant micelles that surround the bioactive component. It has further been discovered that stabilizing the surfactant micelles by adding a biocompatible polymer coats the surfactant micelles to form nanocapsules having a diameter of less than about 50 nm.

As used herein, the term "nanoparticle" refers to a particle having a matrix-type structure with a size of less than about 1,000 nanometers. When the nanoparticle includes a bioactive component, the bioactive component is entangled or embedded in the matrix-type structure of the nanoparticle.

The term "nanosphere", as used herein, refers to a particle having a solid spherical-type structure with a size of less than about 1,000 nanometers. When the nanosphere includes a bioactive component, the bioactive component is adsorbed onto the surface of the nanosphere or embedded in the nanosphere.

Similarly, the term "nanocore", as used herein, refers to a particle having a solid core with a size of less than about 1,000 nanometers. When the nanocore includes a bioactive component, the bioactive component is entangled in the nanocore.
As used herein, the term "nanocapsule" refers to a particle having a hollow core that is surrounded by a shell, such that the particle has a size of less than about 1,000 nanometers. When a nanocapsule includes a bioactive component, the bioactive component is located in the core that is surrounded by the shell of the nanocapsule. The term "nanocapsule" is *not* meant to encompass, and generally does *not* include, a particle having a size of less than about 1,000 nanometers, in which a bioactive component *is* entangled or embedded in the matrix of the nanocapsule or adsorbed onto the surrounding shell of the nanocapsule.

The bioactive component 12 may be included into the first aqueous composition 14 as a liquid, vapor or in granular form. The form of the bioactive component 12 that is selected preferably permits the bioactive component 12 to (1) remain stable prior to dissolving or dispersing into the first aqueous composition 14, (2) be homogeneously dispersed into the first aqueous composition 14, (3) be optionally condensed to reduce a size of the bioactive component 12, (4) be partitioned into the core

of the surfactant micelles 22, (5) be released upon degradation of the biocompatible polymer shell 24 of the nanocapsule 36, and (6) be functionally active upon release from the nanocapsule 36.

The bioactive component 12 may be characterized as "hydrophilic" or "hydrophobic". As used herein, the term "hydrophilic" and "hydrophilicity" refers to an ability of a molecule to adsorb water or form one or more hydrogen-bond(s) with water. All references to "hydrophilic" are also understood as encompassing any portion of the molecule that is capable of adsorbing water or forming one or more hydrogen-bond(s) with water. As used herein, the term "hydrophobic" and "hydrophobicity" refers to an ability of a molecule to not adsorb water nor form one or more hydrogen-bond(s) with water. All references to "hydrophobic" are also understood as encompassing any portion of the molecule that is not capable of adsorbing water nor forming one or more hydrogen-bond(s) with water.

When the bioactive component 12 is a hydrophilic bioactive component, the hydrophilic bioactive component may be directly added to the first aqueous composition 14. As an alternative, the hydrophilic bioactive component 12 may be optionally dissolved or dispersed in one or more solvents, such as water, a nonpolar solvent, a polar solvent, or any combination of any of these.

As used herein, the term "nonpolar solvent" refers to a solvent that does not have a permanent electric dipole moment, and therefore has no ability for an intramolecular association with a polar solvent. Additionally, a nonpolar solvent may be characterized as a solvent that includes molecules having a dielectric constant of less than about 20 units. Similarly, the term "immiscible", as used herein, refers to an inability of two or more substances, such as two or more liquids, solids, vapors, or any combination of any of these, to form an intramolecular association with another substance. Some non-exhaustive examples of nonpolar solvents may be found in Perry's Chemical Engineer's Handbook, Sixth Edition, which is incorporated herein by reference.

As used herein, the term "polar solvent" refers to a solvent that has a permanent electrical dipole moment, and therefore has an ability to form an intramolecular association with another polar substance, such as a liquid, a solid, a vapor or any combination of any of these. Additionally, a polar solvent may be characterized as a solvent that includes molecules having a dielectric constant of more than about 20 units. Likewise, the term "miscible", as used herein, refers to an ability of two or more substances to form an intramolecular association with each other. Some non-exhaustive examples of polar solvents may be found in Perry's Chemical Engineer's Handbook, Sixth Edition, which has been incorporated herein by reference.

When the bioactive component 12 is a hydrophobic bioactive component, the hydrophobic bioactive component may be dispersed or dissolved in a solvent that is capable of dispersing or dissolving the hydrophobic molecule, such as the above-mentioned water, a nonpolar solvent, a polar solvent, or any combination of any of these. Preferably, when the bioactive component 12 is a hydrophobic bioactive component 12, the hydrophobic bioactive component 12 is dissolved or dispersed in a water-miscible solvent, such as, acetone, acetonitrile, ethanol, dimethyl acetamide (DMA), tetrahydrofuran (THF), dioxane, dimethylsulfoxide (DMSO), and dimethylformamide (DMF). Other suitable non-exhaustive examples of water-miscible solvents may be found in Perry's Chemical Engineer's Handbook, Sixth Edition, which has been incorporated herein by reference.

As noted, the bioactive component 12 may be optionally condensed in the first aqueous composition 14 prior to forming the surfactant micelle 16. For example, when the bioactive component is a polynucleotide, the polynucleotide may be condensed using a DNA-condensing agent. As used herein, a "DNA-Condensing Agent" is a molecule that facilitates condensation or a size reduction of DNA.

While condensation of the bioactive component 12 is not critical to the present invention, condensation of the bioactive component 12 may be practiced to reduce the size of the bioactive component 12. Condensation of the bioactive component 12 generally reduces the size of the bioactive component 12 prior to partitioning into the core of the surfactant micelle 16. Reducing the size of the bioactive component 12 may permit maximum incorporation of the bioactive component 12 into the surfactant micelle 22 or may assist a reduction in the overall size of the nanocapsule 36. Increasing the amount of the bioactive component 12 that may be included as part of the nanocapsule 36 permits incorporation of macromolecules having a large number of monomers, such as a large number of base pairs or amino acids, for example. Some non-exhaustive examples of condensing agents have been reviewed in Rolland, A.P. (1998). *Crit. Rev. Therapeutic Drug. Carr. Syst.* 15:143-198, and is incorporated herein by reference.

The bioactive component 12 may further include additional components that are compatible with, and that do not interfere with solvation or dispersion of the bioactive component 12. Some non-exhaustive examples of additional components that may be added to the bioactive component 12 include a DNA-associating moiety, which refers to a molecule, or portions thereof, that interact in a non-covalent fashion with nucleic acids. DNA-associating moieties may include, but are not limited to, a major-and minor-groove binder, a DNA intercalator, a polycation, a DNA-masking component, a membrane-

permeabilizing component, a subcellular-localization component, or the like. Major- and minor-groove binders, as used herein, are molecules thought to interact with DNA by associating with the major or minor groove of double-stranded DNA.

Similarly, the term "DNA intercalator", as used herein, refers to a planar molecule or planar portion of a molecule thought to intercalate into DNA by inserting themselves between, and parallel to, a nucleotide base pair. As used herein, a "polycation" is thought to associate with the negative charges on the DNA backbone. The DNA-associating moiety may be covalently linked through a "reactive group" to a functional component of this invention. The reactive group is easily reacted with a nucleophile on the functional component. Some non-exhaustive examples of reactive groups (with their corresponding reactive nucleophiles) include, but are not limited to N-hydroxysuccinimide (e.g., amine), maleimide and maleimidophenyl (e.g., sulfhydryl), pyridyl disulfide (e.g., sulfhydryl), hydrazide (e.g., carbohydrate), and phenylglyoxal (e.g., arginine).

The term "DNA-masking component", as used herein, refers to a molecule capable of masking all or part of a polynucleotide following release from a nanocapsule to increase its circulatory half-life by inhibiting attack by degrading reagents, such as nucleases, present in the circulation and/or interfering with uptake by the reticuloendothelial system. Similarly, the term "membrane-permeabilizing component", as used herein, refers to any component that aids in the passage of a polynucleotide or encapsulated polynucleotide across a membrane. Therefore, "membrane permeabilizing component" encompasses in part a charge-neutralizing component, usually a polycation, that neutralizes the large negative charge on a polynucleotide, and enables the polynucleotide to traverse the hydrophobic interior of a membrane.

Many charge-neutralizing components can act as membrane-permeabilizers. Membrane-permeabilization may also arise from amphipathic molecules. A "membrane permeabilizer", as used herein, is a molecule that can assist a normally impermeable molecule to traverse a cellular membrane and gain entrance to the cytoplasm of the cell. The membrane permeabilizer may be a peptide, bile salt, glycolipid, phospholipid or detergent molecule. Membrane permeabilizers often have amphipathic properties such that one portion is hydrophobic and another is hydrophilic, permitting them to interact with membranes.

The term "subcellular-localization component", as used herein, refers to a molecule capable of recognizing a subcellular component in a targeted cell. Recognized subcellular components include the nucleus, ribosomes, mitochondria, and chloroplasts. Particular subcellular-localization components include the "nuclear-localization

components" that aid in carrying molecules into the nucleus and are known to include the nuclear localization peptides and amino acid sequences.

The bioactive component 12 may be included at an amount that is therapeutically effective to transform a plurality of cells, such as *in vitro*, *in vivo* or *ex vivo* cells. As used herein, "transform" refers to a presence and/or functional activity of the bioactive component in the plurality of cells after exposing the nanocapsules to the plurality of cells.

Furthermore, those of ordinary skill in the art will recognize that the amount of the bioactive component 12 may vary depending upon the bioactive component 12, the temperature, pH, osmolarity, any solutes, any additional component or optional solvents present in the first aqueous composition 14, the surfactant composition 16, a type or an amount of the surfactant micelle 22, the biocompatible polymer component 24, any desired characteristics of the stabilized surfactant micelle 28, any desired characteristics of the nanocapsules 36, or any combination of any of these.

The bioactive component 12 of the nanocapsule 36 may be supplied as an individual macromolecule or supplied in various prepared mixtures of two or more macromolecules that are subsequently combined to form the bioactive component 12. Some non-exhaustive examples of hydrophilic macromolecules that may be suitable for inclusion as part of the bioactive component 12 include, but are not limited to polynucleotides, polypeptides, genetic material, peptide nucleic acids, aptamers, carbohydrates, mini-chromosomes, molecular polymers, aggregates or associations of an inorganic or organic nature, genes, any other hydrophilic macromolecule or any combination of any of these.

Some non-exhaustive examples of hydrophobic macromolecules that may be included part of the bioactive component 12 include, but are not limited to, adrenergic, adrenocortical steroid, adrenocortical suppressant, aldosterone antagonist, and anabolic agents; analeptic, analgesic, anesthetic, anorectic, and anti-acne agents; anti-adrenergic, anti-allergic, anti-amebic, anti-anemic, and anti-anginal agents; anti-arthritic, anti-asthmatic, anti-atherosclerotic, antibacterial, and anticholinergic agents; anticoagulant, anticonvulsant, antidepressant, antidiabetic, and antidiarrheal agents; antidiuretic, anti-emetic, anti-epileptic, antifibrinolytic, and antifungal agent; antihemorrhagic, inflammatory, antimicrobial, antimigraine, and antimiotic agents; antimycotic, antinauseant, antineoplastic, antineutropenic, and antiparasitic agents; antiproliferative, antipsychotic, antirheumatic, antiseborrheic, and antisecretory agents; antispasmodic, antithrombotic, anti-ulcerative, antiviral, and appetite suppressant agents; blood glucose regulator, bone resorption inhibitor, bronchodilator, cardiovascular, and cholinergic

agents; fluorescent, free oxygen radical scavenger, gastrointestinal motility effector, glucocorticoid, and hair growth stimulant agent; hemostatic, histamine H2 receptor antagonists; hormone; hypocholesterolemic, and hypoglycemic agents; hypolipidemic, hypotensive, and imaging agents, immunizing and agonist agents; mood regulators, mucolytic, mydriatic, or nasal decongestant; neuromuscular blocking agents; neuroprotective, NMDA antagonist, non-hormonal sterol derivative, plasminogen activator, and platelet activating factor antagonist agent; platelet aggregation inhibitor, psychotropic, radioactive, scabicide, and sclerosing agents; sedative, sedative-hypnotic, selective adenosine Al antagonist, serotonin antagonist, and serotonin inhibitor agent; serotonin receptor antagonist, steroid, thyroid hormone, thyroid hormone, and thyroid inhibitor agent; thyromimetic, tranquilizer, amyotrophic lateral sclerosis, cerebral ischemia, and Paget's disease agent; unstable angina, vasoconstrictor, vasodilator, wound healing, and xanthine oxidase inhibitor agent; immunological agents, antigens from pathogens, such as viruses, bacteria, fungi and parasites, optionally in the form of whole inactivated organisms, peptides, proteins, glycoproteins, carbohydrates, or combinations thereof, any examples of pharmacological or immunological agents that fall within the above-mentioned categories and that have been approved for human use that may be found in the published literature, any other hydrophobic bioactive component, or any combination of any of these.

As used herein, the term "polypeptide" refers to a polymer of amino acids not limited by the number of amino acids. It is also to be understood that the term "polypeptide" is meant to encompass an oligopeptide, a peptide, or a protein, for example.

As used herein, the term "polynucleotide" refers to RNA or DNA sequences of more than 1 nucleotide in either single chain, duplex or multiple chain form. The term "polynucleotide" is also meant to encompass polydeoxyribonucleotides containing 2'-deoxy-D-ribose or modified forms thereof, RNA and any other type of polynucleotide which is an N-glycoside or C-glycoside of a purine or pyrimidine base, or modified purine or pyrimidine base or basic nucleotide. The polynucleotide may encode promoter regions, operator regions, structural regions, termination regions, combinations thereof or any other genetically relevant material. Similarly, the term "genetic" as used herein, refers to any material capable of modifying gene expression.

The first aqueous composition 14 may be included in the method of the present invention as a gel, liquid, or in vapor form. The form of the first aqueous composition 14 that is selected preferably permits the first aqueous composition 14 to (1) remain stable prior to dissolving or dispersing the bioactive component, the surfactant composition 16,

the surfactant micelle 22, or optionally the stabilizer surfactant micelle 28, (2) homogeneously disperse the bioactive component 12, the surfactant composition 16, the surfactant micelle 22, or optionally the stabilizer surfactant 28, (3) function as a continuous phase in an oil-in-water emulsion, (4) not interfere with, or mask the functional activity of the bioactive component 12, and (5) not modify or degrade the bioactive component 12, the surfactant composition 16, the surfactant micelle 22, or optionally the stabilized surfactant micelle 28.

The first aqueous composition 14 may include only water, or may optionally include additional solutes or solvents that do not interfere with the method of forming the nanocapsules 36 nor mask the functional activity of the bioactive component 12. Furthermore, those of ordinary skill in the art will recognize that an amount of the first aqueous composition 14 used to prepare the nanocapsules 36 may vary depending upon the bioactive component 12, the surfactant composition 16, the temperature, pH, osmolarity, optional solutes or optional solvents, the surfactant micelle 22, the biocompatible polymer component 24, any desired characteristics of the stabilized surfactant micelle 28 or the nanocapsules 36.

The bioactive component 12 may be added to the first aqueous composition 14 or the first aqueous composition 14 may be added to the bioactive component 12. While the order of addition of the bioactive component 12 and the first aqueous composition 14 is not critical to the present invention, the hydrophilic composition (not shown) that is formed when the bioactive component 12 is dissolved or dispersed in the first aqueous composition 14 is preferably capable of maintaining a homogeneous solution or dispersion in the hydrophilic composition.

The first aqueous composition 14 may be supplied as an individual component or supplied in various prepared mixtures of two or more components that are subsequently combined to form the first aqueous composition 14. Some non-exhaustive examples of the first aqueous composition 14 include, but are not limited to, the above-mentioned water, nonpolar solvents, polar solvents, or any combination of any of these. Preferably, water is the first aqueous composition 14.

The surfactant composition 16 may be introduced into the bioactive component 12, the first aqueous composition 14, the hydrophilic composition as a liquid, vapor or in granular form. The form of the surfactant composition 16 that is selected preferably permits the surfactant composition 16 to (1) remain stable prior to introducing into the bioactive component 12, the first aqueous composition 14, or the hydrophilic composition, (2) be homogeneously dispersed into the bioactive component 12, the first aqueous composition 14, or the hydrophilic composition, (3) form a micellar structure,

(4) be adsorbed onto a surface of the bioactive component 12, the first aqueous composition 14, the hydrophilic composition (5) displace the first aqueous composition that is located on the surface of the bioactive component 12, (6) partition the bioactive component 12 or the hydrophilic composition into a core of the micellar structure to form the surfactant micelle 22, and (7) provide a thermodynamic driving force that is effective to reduce a size of the bioactive component 12, surfactant micelle 22, the stabilized surfactant 28 or the nanocapsule 36.

As used herein, a "surfactant" refers to any molecule containing a polar portion that thermodynamically prefers to be solvated by a polar solvent, and a hydrocarbon portion that thermodynamically prefers to be solvated by a non-polar solvent. The term "surfactant" is also meant to encompass anionic, cationic, or non-ionic surfactants. As used herein, the term "anionic surfactant" refers to a surfactant with a polar portion that ionizes to form an anion in aqueous solution. Similarly, a "cationic surfactant" refers to a surfactant having a cationic polar portion that ionizes to form a cation in aqueous solution. Likewise, a "non-ionic" surfactant refers to a surfactant having a polar portion that does not ionize in aqueous solution.

While not wanting to be bound to theory, it is generally believed that a surfactant refers to a molecule that is effective to reduce a surface or an interfacial tension between a first substance dispersed in a second substance such that the first substance is solvated and any molecular groups of the first substance are dispersed. Typically, a hydrodynamic diameter of the first substance increases after addition of the surfactant. Nonetheless, the surfactant composition 16 is believed to be effective to *reduce the size or diameter* of the surfactant micelles 22 in the first aqueous composition 14, to thereby reduce the size of the nanocapsule 36 when practicing the present invention.

The surfactant composition 16 may include the surfactant component only (not shown), or may optionally include the biocompatible oil component 18. The surfactant component may be characterized on the HLB (Hydrophile-Lipophile Balance) scale that ranges from less than about 1 to more than about 13 units.

A surfactant component having an HLB value of less than about 6.0 units may be described as being poorly, or not dispersable in an aqueous or water-based composition. In addition, a surfactant component having an HLB value of less than about 6.0 units may be characterized as a hydrophobic or non-ionic surfactant. A surfactant component having an HLB value of more than about 7.0 units may be described as being

capable of forming a milky to translucent to clear dispersion when the surfactant having an HLB value of more than about 7.0 units is dispersed in an aqueous or water-based composition.

Preferably, the surfactant component of the surfactant composition 16 has an HLB value of less than about 6.0 units when practicing the method of the present invention. Still more preferably the surfactant component of the surfactant composition 16 has an HLB value of less than about 5.0 units to facilitate preparation of nanocapsules having a diameter of less than about 50 nm.

The surfactant component may also be characterized in terms of a critical micelle concentration (CMC) value. Preferably, the surfactant component of the surfactant composition 16 has a CMC value of less than about 300 micromolars (μm). Still more preferably, the surfactant component has a CMC value of less than about 200 μm.

While not wanting to be bound to theory, it is believed that the surfactant component of the surfactant composition 16 adsorbs onto the surface of the bioactive component 12 when introduced into the first aqueous composition 14 to minimize exposure of a surface of the hydrophobic surfactant component to a thermodynamically unfavorable environment created by the first aqueous composition 14. Therefore, the surfactant component adsorbs onto the surface of the bioactive component to reduce the surface area of the surfactant component that may be exposed to the first aqueous composition 14. Adsorption of the surfactant component onto the bioactive component 12 is believed to facilitate the size reduction of the bioactive component 12 and/or the surfactant micelle 22.

The surfactant component of the surfactant composition 16 may be supplied as individual surfactants or supplied in various prepared mixtures of two or more surfactants that are subsequently combined to form the surfactant composition 16. Some non-exhaustive examples of suitable surfactants having an HLB value of less than about 6.0 units or a CMC value of less than about 200 μm be listed in *Dermatological Formulations (Barry, B., Marcel Dekker, (1983))*, or in *Percutaneous absorption: drug, cosmetics, mechanisms, methodology, 3rd ed., Bronough, R. ed., 1999*, or the *Handbook of Industrial Surfactants (Ash, M., Ed., Gower Pub. (1993)*, which are incorporated herein by reference. As an example, the surfactant component may be 2, 4, 7, 9-tetramethyl-5-decyn-4, 7-diol(TM-diol), blends of 2, 4, 7, 9-tetramethyl-5-decyn-4, 7-diol(TM-diol), molecules having one or more acetylenic diol groups, cetyl alcohol or any combination of any of these.

The optional biocompatible oil component 18 of the surfactant composition 16 may be combined with the surfactant component as a liquid, vapor or in granular form. The form of the optional biocompatible oil component 18 that is selected preferably permits the optional biocompatible oil component 18 to (1) remain stable prior to introduction into the surfactant composition 16, (2) be homogeneously blended into the surfactant composition 16, (3) dissolve or disperse the surfactant component, and (4) increase the hydrophobicity of the surfactant composition 16, and therefore, the degree to which the size of the bioactive component 12, the surfactant micelle 22, the stabilizer surfactant micelle 28, or the nanocapsule 36 may be reduced when practicing the present invention.

Preferably, the concentration of the optional biocompatible oil component 18 in the surfactant composition 16 ranges from about 10^{-7} weight percent to about 10 weight percent, based upon a total volume of the stabilized surfactant micelles 28. Concentrations of the optional biocompatible oil component 18 higher than about 10 weight percent, based upon the total volume of the stabilized surfactant micelles 28, may be less desirable because such higher concentrations increase a phase volume of the biocompatible oil, and consequently may cause difficulties in preparing, dispersing and/or handling the surfactant micelles 22, the stabilized surfactant micelles 28 or the nanocapsules 36. Concentrations of the optional biocompatible oil component lower than about 10^{-7} weight percent in the surfactant composition 16 may be less preferred, because such lower concentrations would not be effective to solvate the surfactant component, or increase the hydrophobicity of the surfactant composition 16, and may ultimately *increase* the diameter of the nanocapsules 36.

The optional biocompatible oil component 18 of the surfactant composition 16 may be supplied as an individual biocompatible oil or supplied in various prepared mixtures of two or more biocompatible oils that are subsequently combined to form the optional biocompatible oil component 18. Some non-exhaustive examples of suitable biocompatible oils that may be included as part of the biocompatible oil component 18 may be found in *Dermatological Formulations (Barry, B., Marcel Dekker, (1983))*, or in *Percutaneous absorption: drug, cosmetics, mechanisms, methodology, 3rd ed., Bronough, R. ed. , 1999*, or in the *Handbook of Industrial Surfactants (Ash, M., Ed., Gower Pub. (1993)*, which have been incorporated herein by reference. Preferably, food or USP grade oils, such as DMSO, DMF, castor oil, or any combination thereof, are used to practice the present method.

The surfactant composition 16 may be included at an amount that is effective to form the micellar structure that partitions the bioactive component 12, the first aqueous composition 14 or the hydrophilic composition into the core of the micellar structure when forming the surfactant micelle 22. Still more preferably, the surfactant composition 16 is included at an amount that is effective to provide a maximum thermodynamic driving force that minimizes the size of the bioactive component 12, the surfactant micelle 22, and ultimately, the size of the nanocapsule 36 when practicing the present invention.

Furthermore, those of ordinary skill in the art will recognize that the amount of the surfactant composition 16 may be varied based upon the bioactive component 12, the first aqueous composition 14, a ratio of the surfactant component to the optional biocompatible oil 18, any desired characteristics of the surfactant micelles 22, the stabilized surfactant micelles 28 or the nanocapsules 36. For example, a surfactant composition containing a surfactant component having an HLB value of about 6.0 units mixed with a nonpolar biocompatible oil like castor oil, may provide the same degree of a thermodynamic driving force as a second surfactant composition containing a surfactant component of about 4.0 units mixed with DMSO.

The amount of the surfactant composition 16 may range up to about 0.5 weight percent, based upon a total volume of the stabilized surfactant micelles 28. Still more preferably, the amount of the surfactant composition 16 is less than about 0.25 weight percent, based upon the total volume of the stabilized surfactant micelles 28. Most preferably, the surfactant composition 16 is present at an amount of less than about 0.05 weight percent, based upon the total volume of the stabilized surfactant micelles 28. As one non-exhaustive example, the surfactant composition 16 may be added to the total volume of the hydrophilic composition at a concentration of about 500 ppm, based on the total volume of the stabilized surfactant micelles 28.

The first dispersing apparatus 20 initiates and promotes formation of the micellar structures that are based on the bioactive component 12, the first aqueous composition 14 and the surfactant composition 16. Adsorption of surfactant component onto the surface of the bioactive component 12, or hydrophilic composition continues until all of the surfactant molecules cover, and therefore, entrap the bioactive component 12 or hydrophilic composition in the core of the micellar structure to form surfactant micelles 22. Formation of a plurality of surfactant micelles 22 in the first aqueous composition 14 forms a dispersion of surfactant micelles 22.

In general, any conventional dispersing apparatus 20 that is capable of homogenously blending or dispersing may be suitable for use in forming the dispersion

of surfactant micelles in accordance with the present invention. Furthermore, those of ordinary skill in the art will recognize that the first dispersing apparatus 20 may vary depending upon the desired characteristics of the nanocapsules 36. For example, the first dispersing apparatus 20 may include any device, such as a sonicating or a vortexing apparatus (not shown), or the like to disperse the bioactive component 12 in the hydrophilic composition, and the formation of the surfactant micelles 22 after addition of the surfactant composition 16. Nonetheless, while the first dispersing apparatus 20 may include a sonicating or a vortexing apparatus, the sonicating or the vortexing apparatus is not critical when practicing the method of the present invention.

As used herein, a "surfactant micelle" may be characterized as a close packed mono-molecular barrier of surfactant molecules at an interface between the bioactive composition 12 and the surfactant composition 16, such that the barrier encapsulates the bioactive component 12, the first aqueous composition 14 or the hydrophilic composition. It is also to be understood that the term "surfactant micelle" encompasses partial or hemi-surfactant micelles that partially enclose the bioactive component 12, the first aqueous composition 14 or the hydrophilic composition.

When the bioactive component 12 is a hydrophilic bioactive component, the polar portion of the surfactant molecule associates with a surface of the hydrophilic bioactive component. When the bioactive component 12 is a hydrophobic bioactive component, the hydrocarbon portion of the surfactant micelle associates with a surface of the hydrophobic bioactive component.

The formation of a surfactant micelle typically occurs at a well defined concentration known as the critical micelle concentration. As noted, surfactant components having a CMC value of less than about 200 micromolars are preferred when practicing the present invention.

After forming the dispersion of surfactant micelles 22, the dispersion of surfactant micelles 22 is transferred into the stabilizing apparatus 26 where a biocompatible polymer component 24 is added to stabilize the dispersion of surfactant micelles 22. Alternatively, the biocompatible polymer component 24 may be added to the dispersion of surfactant micelles 22 in the first dispersing apparatus 20 which obviates the need for the stabilizing apparatus 26.

The biocompatible polymer component 24 stabilizes the dispersion of surfactant micelles 22 to form stabilized surfactant micelles 28 within the first aqueous composition 14. Therefore, a dispersion of stabilized surfactant micelles 28 are present within the first aqueous composition 14 after addition of the biocompatible polymer component 24.

As used herein, the term "biocompatible" refers to a material that is capable of interacting with a biological system without causing cytotoxicity, undesired protein or nucleic acid modification or activation of an undesired immune response.

The biocompatible polymer component 24 may be introduced into the dispersion of surfactant micelles 22 as a liquid, vapor or in granular form. The form of the biocompatible polymer component 24 that is selected preferably permits the biocompatible polymer component 24 to (1) remain stable prior to addition into the dispersion of surfactant micelles 22, (2) be homogeneously dispersed into the dispersion of surfactant micelles 22, (3) increase a viscosity of the first aqueous composition 14, (4) form a boundary layer at an interface of the surfactant micelle 22 and the first aqueous composition 14, (5) be absorbed onto a surface of the surfactant micelles 22, (6) be capable of iontophoretic exchange, (7) be capable of being precipitated upon addition of a solute, (8) be capable of enzymatic degradation, surface and/or bulk erosion, (9) not interfere with or mask the functional activity of the bioactive component 12, (10) prevent aggregation and/or agglomeration of the dispersion of surfactant micelles 22, and (11) be capable of obtaining a particular dissolution profile.

The biocompatible polymer component 24 may be included at an amount that is effective to coat and therefore stabilize the surfactant micelle 22. Furthermore, those of ordinary skill in the art will recognize that the amount of the biocompatible polymer component 24 used to stabilize the surfactant micelles 22 may vary depending upon the bioactive component 12, the first aqueous composition 14, the surfactant composition 16, the temperature, pH, osmolarity, presence of any optional solutes or optional solvents, the surfactant micelle 22, any desired characteristics of the stabilized surfactant micelle 28, the nanocapsules 36, or a desired dissolution profile.

While the concentration of the biocompatible polymer component 24 is not critical to the present invention, the concentration of the biocompatible polymer component 24 is preferably based upon the bioactive component 12 and on the desired dissolution profile. When the concentration of the biocompatible polymer component 24 is too high, the shell of the nanocapsule 36 may not dissolve. If the concentration of the biocompatible polymer component 24 is too low, the shell of the nanocapsule 36 may dissolve rapidly in a manner that promotes cytotoxicity, for example. In addition, too low a concentration of the biocompatible polymer component 24 may not provide an effective degree of mechanical force to stabilize the surfactant micelles 28.

Concentrations of the biocompatible polymer component 24 that are too high may also be less desirable because such higher concentrations may increase the viscosity of the first aqueous composition 14, and consequently may cause difficulties in preparing,

mixing and/or transferring the stabilizer surfactant micelles 28. Concentrations of the biocompatible polymer component 24 that are too low may be less preferred, because lower concentrations may not provide the needed viscosity to stabilize the surfactant micelles 22, nor be capable of effectively coating the surfactant micelles 22 to prevent aggregation of the surfactant micelles 22 in the first aqueous composition 14.

The biocompatible polymer component 24 may be supplied as individual biocompatible polymers or supplied in various prepared mixtures of two or more biocompatible polymers that are subsequently combined to form the biocompatible polymer component 18. Some non-exhaustive examples of biocompatible polymers include polyamides, polycarbonates, polyalkylenes, polyalkylene glycols, polyalkylene oxides, polyalkylene terepthalates, polyvinyl alcohols, polyvinyl ethers, polyvinyl esters, polyvinyl halides, polyvinylpyrrolidone, polyglycolides, polysiloxanes, polyurethanes and copolymers thereof, alkyl cellulose, hydroxyalkyl celluloses, cellulose ethers, cellulose esters, nitro celluloses, polymers of acrylic and methacrylic esters, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, hydroxy-propyl methyl cellulose, hydroxybutyl methyl cellulose, cellulose acetate, cellulose propionate, cellulose acetate butyrate, cellulose acetate phthalate, carboxylethyl cellulose, cellulose triacetate, cellulose sulphate sodium salt, poly(methylmethacrylate), poly(ethylmethacrylate), poly(butylmethacrylate), poly(isobutylmethacrylate), poly(hexlmethacrylate), poly(isodecylmethacrylate), poly(laurylmethacrylate), poly(phenylmethacrylate), poly(methacrylate), poly(isopropacrylate), poly(isobutacrylate), poly(octadecacrylate), polyethylene, polypropylene poly(ethylene glycol), poly(ethylene oxide), poly(ethylene terephthalate), poly(vinyl alcohols), poly(vinyl acetate), poly vinyl chloride, polystyrene, polyhyaluronic acids, casein, gelatin, gluten, polyanhydrides, polyacrylic acid, alginate, chitosan, any copolymers thereof, and any combination of any of these.

Additionally, biocompatible polymers that have been modified for desirable enzymatic degradation, or change upon application of light, ultrasonic energy, radiation, a change in temperature, pH, osmolarity, solute or solvent concentration may also be included as part of the biocompatible polymer component 24. Preferably, the biocompatible polymer component 24 is a hydrophilic polymer that is capable of substantially coating, and preferably continuously coating the surfactant micelle 22. Still more preferably, the hydrophilic biocompatible polymer component 24 is capable of ionotophoretic exchange.

Though descriptions of the present invention are primarily made in terms of a hydrophilic biocompatible polymer component 24, it is to be understood that any other biocompatible polymer, such as hydrophobic biocompatible polymers may be substituted

in place of the hydrophilic biocompatible polymer, in accordance with the present invention, while still realizing benefits of the present invention. Likewise, it is to be understood that any combination of any biocompatible polymer may be included in accordance with the present invention, while still realizing benefits of the present invention.

In general, any conventional apparatus and technique that is suitable for permitting the biocompatible polymer component 24 to stabilize the surfactant micelles 22 may be used as the stabilizing apparatus 26 in accordance with the present invention. Furthermore, any other device, such as high pressure homogenization or high ultrasound sonication is preferably not included during stabilization.

After stabilizing the surfactant micelles 22, the stabilized surfactant micelles 28 may be transferred into a second aqueous composition 30 located in a second dispersing apparatus 32. The stabilized surfactant micelles 28 may be transferred by mechanically forming droplets of the stabilized surfactant micelle 28 that are subsequently introduced into the second aqueous composition 30.

The second aqueous composition 30 may include water only, or may optionally include a solute to precipitate the biocompatible polymer component 24 surrounding the stabilized surfactant micelle 28. Some non-exhaustive examples of solutes that may be used to precipitate the biocompatible polymer 24 include ionic species derived from elements listed in the periodic table.

Preferably, the second aqueous composition 30 includes a solute in an amount that is effective to precipitate the biocompatible polymer component 24 and form the dispersed, and optionally atomized nanocapsules 36 of the present invention. As used herein, the term "precipitate" refers to a solidifying or a hardening of the biocompatible polymer component 24 that surrounds the stabilized surfactant micelles 28. It is also to be understood that the term "precipitation" is also meant to encompass any crystallization of the biocompatible polymer 24 that may occur when the biocompatible polymer component 24 is exposed to the solute.

Additionally, any other component that is capable of modulating the efficacy the nanocapsules 36 may be included as part of the second aqueous composition to thereby modulate the functional activity of the nanocapsule 36. For example, the second aqueous composition may include additional coating excipients, such as a cell recognition component or various ionic species, such as Mn^{2+}, Mg^{2+}, Ca^{2+}, Al^{3+}, Be^{2+}, Li^{+}, Ba^{2+}, Gd^{3+}, or any other ionic species that is capable of interacting with the biocompatible polymer component 24.

The term "cell recognition component", as used herein, refers to a molecule capable of recognizing a component on a surface of a targeted cell. Cell recognition components may include an antibody to a cell surface antigen, a ligand for a cell surface receptor, such as cell surface receptors involved in receptor-mediated endocytosis, peptide hormones, and the like.

It has been observed that when the stabilized surfactant micelles 28 are allowed to incubate in the second aqueous composition 30 that includes the solute to precipitate the biocompatible polymer component 24, the nanocapsules 36 undergo a reduction in size. Furthermore, the formation of a flocculated suspension of the nanocapsules 36 has also been observed after incubating the stabilized surfactant micelles 28 in the second aqueous composition.

As used herein, "a flocculated suspension" refers to the formation of a loose aggregation of discrete particles held together in a network-like structure either by physical absorption of bioactive components, bridging during chemical interaction (precipitation), or when longer range van der Waals forces of attraction exceed shorter range forces of repulsion. The flocculated suspension of nanocapsules 36 may entrap varying amounts of the first aqueous composition 14 or the second aqueous composition 30 within the network-like structure. Additionally, the flocculated suspension of nanocapsules may be gently tapped to disperse the nanocapsules 36.

The stabilized surfactant micelles 28 may be transferred into the second aqueous composition 30 via atomization through a nozzle (not shown) having a particular orifice size or through an aerosolizing apparatus (not shown). Atomizing or aerosolizing the stabilized surfactant micelles 28 typically includes the application of a shear force that may be capable of further dispersing the stabilized surfactant micelles 28. Furthermore, the application of the shear force during transfer may also be effective to (1) reduce the size of the nanocapsules 36, or (2) break up any agglomerates or associations between stabilized surfactant micelles 28 that may have formed in the stabilizing apparatus 26. Feed pressures of less than about 100 psi, for example, may be used to atomize the stabilized surfactant micelles 28.

The diameter of the nanocapsules 36 may also be varied depending upon the orifice size of the nozzle that may be used to transfer the stabilized surfactant micelles 28 into the second aqueous composition. Alternatively, the stabilized surfactant micelles 28 may be added to the second aqueous composition 30 containing the solute that precipitates the biocompatible polymer 24 to form a dispersion of nanocapsules 36 for purposes of providing the dispersion for sub-cutaneous delivery of the nanocapsules, for example.

After precipitating and/or optionally incubating the nanocapsules 36 in the second aqueous composition 30, the nanocapsules 36 may be filtered, centrifuged or dried to obtain separate and discrete nanocapsules 36. The nanocapsules 36 may be frozen or reconstituted for later use or may be delivered to a target cell or tissue by such routes of administration as oral, intravenous, subcutaneous, intraperitoneal, intrathecal, intramuscular, inhalational, topical, transdermal, suppository (rectal), pessary (vaginal), intra urethral, intraportal, intrahepatic, intra-arterial, intra-ocular, transtympanic, intratumoral, intrathecal, transmucosal, buccal, or any combination of any of these.

The nanocapsules 36 having a diameter of less than about 50 nm are advantageous in the delivery of bioactive components to target cells for several reasons. First, nanocapsules 36 having a diameter of less than about 50 nm enhances delivery of bioactive components by protecting the bioactive components against degradation during transport to the target cell.

Second, nanocapsules 36 having a diameter of less than about 50 nm promotes efficient cellular uptake. Efficient cellular uptake *into* the target cell typically occurs when a particle has a diameter of less than about 50 nm, as opposed to when a particle has a diameter of more than about 50 nm.

Third, it is believed that uptake of the nanocapsules 36 by the target cell occurs via transport systems, such as a non-endosomal pathway, that prevents lysosomal degradation of the nanocapsules 36. Indeed, it is believed that the nanocapsules 36 of the present invention are efficiently exported *into* a cell via a caveolin-regulated pathway that circumvents most, if not all, endosomal-regulated pathways that typically degrade nanocapsules 36.

Fourth, the nanocapsules 36 have a biocompatible polymer shell that is separate from the bioactive component. In fact, the bioactive component is *not* entangled in, embedded in, or adsorbed onto the biocompatible polymer shell of the nanocapsules 36. When the bioactive component is not entangled in, embedded in, or adsorbed onto the biocompatible polymer shell, the cell that incorporate the nanocapsules 36 avoid apoptosis or cell death.

Fifth, enclosing the bioactive component within a core surrounded by the biocompatible polymer shell when preparing the nanocapsules 36 in accordance with the present method is advantageous in avoiding premature degradation of the nanocapsules 36, or a cytotoxic response during *in vivo* transport of the nanocapsule. Enclosing the bioactive component within the core results in a linear release rate of the bioactive component without any zero burst phenomenon during release from the nanocapsules 36.

The linear release rate of the bioactive component from the nanocapsule *without* any zero burst phenomenon is also an advantageous feature as the linear release rate allows rational design of coating dissolution profiles to minimize cytotoxicity. As used herein, the term "dissolution profile" refers to a rate at which the biocompatible polymer shell is dissolved or degraded to release a bioactive agent from a core of a nanocapsule.

Another benefit of the nanocapsules 36 prepared by the method of the present invention is that little, if any, addition of an organic solvent is required to form the nanocapsules 36. Eliminating the use of most, if not all, organic solvents from the method of the present invention is beneficial since organic solvents may damage the bioactive component 12, destroy the target cells, or be toxic during preparation of the nanocapsule 36. The elimination of most, if not all, use of organic solvents eliminates the need for complex solvent removal techniques, such as solvent dilution, vacuum evaporation, or the like, and obviates any associated costs or complex process strategies during preparation of the nanocapsules 36.

The nanocapsules 36 of the present invention further permits stable encapsulation of a bioactive component, and in particular, hydrophilic bioactive components, such as polynucleotides and polypeptides. "Stable encapsulation", as used herein, refers to maintenance of the encapsulated bioactive component's structure. For nucleic acids, the appearance of low molecular weight nucleic acid breakdown products, which may be assayed for by electrophoresis, is substantially eliminated. The nanocapsules 36 may also be used to encapsulate any bioactive component regardless of water solubility or charge density.

APPLICATIONS

The nanocapsules 36 may be combined with additional polymeric binders, surfactants, fillers, and other excipients to incorporate the nanocapsules 36 into solid dosage forms such as granules, tablets, pellets, films or coatings for use in enhanced bioactive component 12 delivery. In this way, design of the dissolution profile, control of the particle size, and cellular uptake remains at the level of the nanocapsule. Such applications include, but are not limited to, creation of rapidly dissolving pellets of nanocapsules for pulmonary delivery or nanocapsule films for device-mediated delivery.

In another application, the nanocapsules 36 may be designed for specific cellular or tissue uptake by polymer selection and/or inclusion of cell-recognition components in the nanocapsule biocompatible polymer shell or coating. Such coatings will have utility for specific or increased delivery of the bioactive agent to the target cell. Such applications include, but are not limited to tumor-targeting of chemotherapeutic

agents or anti-sense DNA, antigen delivery to antigen-presenting cells, ocular delivery of ribozymes to retinal cells, transdermal delivery of protein antibodies, or transtympanic membrane delivery of peptide nucleic acids.

PROPERTY DETERMINATION AND CHARACTERIZATION TECHNIQUES

Various analytical techniques are employed herein. An explanation of these techniques follows:

FIG. 2A: Samples were prepared on freshly cleaved mica as dispensed, dried in air and imaged using a Nanoscope II multimode AFM (Digital Instruments) with a J type scanner and ambient tapping mode holder. 125 μm long silicon cantilevers type IBMSC were from IBM and have resonant frequencies of 250 - 450 kHz. All imaging was in tapping mode, images were 512 x 512 pixels and scanning frequency was 1 kHz. Height, amplitude and phase images were collected. Images were processed in DI software and analyzed in NIH Image SXM. A: Formula Q from 2-phase system, low HLB surfactant, B: Formula S from 2-phase system, high HLB surfactant, C: Formula T from 1-phase system, high HLB surfactant, D: Formula V from 2-phase system, surfactant below CMC.

FIG. 2B: Nanocapsules were released into a solution of 10% isobutanol in Phosphate-buffered Saline (PBS), pH=7.2. Samples were run in duplicate.

FIG. 2C: Nominal 300 ng samples of DNA were aliquoted from a master batch containing surfactant and processed through commercial miniprep columns. Eluate was recycled through Qiaquik™ columns and collected either 3 times (4, 5) or twice (6,7) or recycled through Zymoclean™ columns and collected twice (8,9). Samples were alcohol precipitated using a commercial coprecipitant, electrophoresed on 1.5% agarose gels modified with Synergel™, stained with SybrGold™ dye, digitized on a Storm 860™ and compared to unmodified but reprecipitated samples from the same master batch (10,11). Lanes 1-3: 100, 50 and 5 ng of lambda-DNA.

FIG. 3: Endocytic activity was assessed by immunosignal levels of clathrin (Chemicon). Potocytotic activity was assessed by immunosignal for caveolin-1 as described in the literature (Transduction Laboratories). Lysosomal activity was detected by an monoclonal antibody to Lamp-1 (Transduction Laboratories). Nanocapsule localization was detected by streptavidin-biotin immunocomplexes directed against sheep IgG (Jackson Laboratories). Nanocapsule coatings were spiked with ovine IgG to enable this detection strategy.

FIG. 4: Immortalized Rt-1 fibroblast cultures at 70% confluence were treated for 4 days with increasing amounts of nanocapsule formula K and transiently treated (3

hours) with an optimized liposomal formula (dose, 500 ng) Results are expressed as a percentage of cellular actin integrated intensity and compared to the liposomal formula. Expression vector was code 448: pEF/myc-his/GFP (Invitrogen).

FIG. 5A: Radiated porcine biopsies were snapfrozen 7 days after treatment with saline or 6 μg of controlled release nanocapsules, then homogenized in RIPA. 100 μg lysate samples were electrophoresed on SDS-Page gradient gels, transferred to nitrocellulose membranes and detected for either beta-galactosidase (about 121 kilo Dalton (kD)) or involucrin (about 100 kD) using chemiluminescence. Results were normalized to the post-transfer gel stained with Coomassie due to interference at 100 kD from a gel defect. Involucrin, a component of the cornified membrane, manufactured by suprabasal cells can be detected in radiated porcine skin and will be used for future normalization purposes. Lane A: N, topical, biopsy oc-2; B: N, topical, biopsy oc-3; C: O, topical, biopsy 1-1; D: PBS only, biopsy 1-5; E: N, subcutaneous injection, biopsy 1-6.

FIG. 5B: The beta-galactosidase reporter protein was detected by a monoclonal antibody directed at an incorporated fusion protein tag. A: N, topical, biopsy oc-1, detection with anti-Xpress™; B: Matching view to A with detection for anti-von Willenbrand factor (Sigma);C: untreated biopsy, detection with anti-Xpress™ (Invitrogen).

FIG. 6: Nanocapsules were incorporated into an aqueous suture coating and sutures were applied to pigskin biopsies in organ culture. Nanocapsules were detected with Cy3 conjugated-streptavidin-biotin complexes to incorporated ovine IgG and nuclear localized GFP transgene expression was detected by rabbit polyclonal antibodies to GFP (Abcom) in combination with Fitc-conjugated polyclonal antibodies to rabbit IgG and Alexa 488-conjugated polyclonal antibodies to Fitc (Molecular Probes). Cell nuclei were counterstained with 10 μg/ml bisbenzamide. Controls omitting primary antibodies were included for specificity determination and signal-to-background level estimation.

FIG. 7A: Nanocapsules were detected as previously described and nuclear-localized GFP transgene expression was detected by rabbit polyclonal antibodies to GFP in combination with Cy3- conjugated antibodies to rabbit IgG (Jackson Laboratories).

FIG. 7B: GFP expression was detected as described in FIG. 6 and cell nuclei were counterstained with 10 μg/ml bisbenzamide.

FIG. 7C: Carcinoma cells and HDF's were seeded overnight into 96 well plates at 2000 and 6000 cells per well respectively. Cisplatin preparations were added to wells for 18 hours as noted on the graph than washed out. After 72 hours, cell viability

was assessed by a commercial MTT assay (WST assay, Boehringer Mannheim). Wells were executed in duplicate.

FIGS. 8A and 8B: Colocalization with lysosomes was detected using a monoclonal antibody to Lamp-1 (Transduction Laboratories). AFM images are included of O-methyl RNA formulated by nanoencapsulation or complexation with 27 kD polyethyleneimine.

EXAMPLES

The present invention is more particularly described in the following Examples which are intended as illustrations only since numerous modifications and variations within the scope of the present invention will be apparent to those skilled in the art.

Reagents:

A. Nucleic acid condensing agents

Poly(ethylenimine) (PEI) at 27 KiloDalton (kD). PEI was used at optimized conditions (90% charge neutralization)

Polylysine (PLL) at 70-150,000 molecular weight. PLL condensing materials were conjugated with nuclear signal localization peptides, either SV-40 T antigen or cys-gly-tyr-gly-pro-lys-lys-lys-arg-lys-val-gly-gly using carboxiimide chemistry available from Pierce Chemical (Rockford, IL).

Preparations of nuclear matrix proteins (NMP). NMP were collected from a rat fibroblast cell line, and a human keratinocyte cell line using a procedure described in Gerner et al. *J. Cell. Biochem.* 71 (1998):363-374 which is incorporated herein by reference. Protein preparations were conjugated with nuclear signal localization peptides as described.

B. Surfactants

2, 4, 7, 9 - tetramethyl-5-decyn-4, 7 - diol (TM-diol): HLB = 4-5, CMC is not determined

Poly(oxy-1, 2-ethanediol), a-(4-nonylphenol)-w-hydroxy, Tergitol NP-40 (NP40), Nonoxynol-40, POE (40) nonyl phenyl ether: HLB=17.8, CMC 232 μM,

Polyoxyethylene 80 sorbitan monooleate (Tween 80): HLB = 10, CMC 12 μM,

Cetyl Alcohol: HLB = 4,CMC is not determined.

C. Polymers

Hyaluronan, recombinant, 1 million kiloDalton (MM kD) and conjugated with nuclear localization signal peptides as described in U.S. Patent 5,846,561, which is incorporated herein by reference.

Hyaluronan, derived from human umbilical cord, about 4MM kD and not conjugated.

Povidone (polyvinylpyrolidone, PVP) 10,000 kD MW and not bioconjugated.

Povidone (polyvinylpyrolidone, PVP) 40,000 Kd MW and not bioconjugated.

Povidone (polyvinylpyrolidone, PVP) 360,000 kD MW and not bioconjugated.

Tenascin, 220 kD and not bioconjugated.

D. Expression Vectors

334 : pcDNA/His/LacZ, produces galactosidase, incorporates CMV promoter, based on pcDNA 3.1. (Invitrogen), 8.6 kilobases (kB).

425 : pEGFP-c/farn, enhanced GFP (green fluorescent protein) expression vector modified with a farnasyl moiety to improve microscopy, CMV promoter, 4.6 kB

423 : pEGFP-c3/p57(Kpn/Sma) Clontech enhanced GFP (green fluorescent protein) expression vector modified with a nuclear localization tag from a cyclin dependent kinase to improve microscopy, 4.6 kB

E. Cells

CCRL 1764: Immortalized rat neonatal fibroblast cell line (RT-1's)

HaCaT: immortalized human keratinocyte cell line

Ca9: human tumor cells derived from a squamous cell carcinoma of tongue origin.

Example 1A - Effect of changing dispersion conditions on hydrophillic nanocapsules.

The importance of appropriate dispersion conditions was investigated in the following series of formulations. Formulae were produced by i) predispersing 25 μg of DNA (425) on ice using a bath sonicator, ii) condensing DNA in a small amount of water by vortexing then incubating on ice for 20 minutes, iii) adding surfactant then oil followed by 30 seconds of probe sonication at 10 Watts, iv) dispersion dilution to 3 milliliters (mL) by first adding saline then 1 MM kD hyaluronan polymer (1%) as a protective colloid with bath sonication, v) mechanically shearing emulsion into droplets by pumping through a 250 micrometer (μm) orifice into 22 mL of PBS, 10 millimolar (mM) Ca^{2+}, 200 mM Li^{+}, vi) incubating overnight end over end and vii) centrifuging to recover nanoparticles for resuspension and filter sterilization. The condenser-to-DNA weight ratio was determined by dye exclusion at 90% charge neutralization. TM-diols were used in this experiment to represent water-immiscible surfactants, while Tergitol NP40 and Tween 80 were used to represent water-soluble and even more water-soluble emulsifiers/dispersing aids.

Dispersion conditions were systematically varied to discourage micelle formation in aqueous media by i) choosing water-soluble surfactants (Formulae S,T,U,W and V), ii) removing the dispersed phase (Formula T) and iii) decreasing surfactant loading below that required for micelle formation. Formula U featured use of a water-immiscible oil (silicone oil). Formulas were characterized physically and tested for functionality in *in vitro* gene transfer. Quantitative results are summarized in Table 1A:

Table 1A: Effect of changing dispersion conditions on hydrophillic nanocapsules.

Formula	*Q*	*R*	*S*	*T*	*U*	*W*	*V*
Experimental Modification:	surf > CMC	surf > CMC	surf > CMC	surf > CMC	surf>CMC	surf > CMC	surf < CMC
Critical Micelle Concentration (CMC)	~ 0	~ 0	460 ppm	460 ppm	460 ppm	15 ppm	460 ppm
Pre-aerosol surfactant Concentration (3 ml basis)	500 ppm	500 ppm	600 ppm	600 ppm	600 ppm	4000 ppm	90 ppm
HLB number	4 - 5	4	17.8	17.8	17.8	10	17.8
Phases	Water/misc. oil	Water/misc. oil	Water/misc. oil	Water only	Water/immisc. oil	Water/misc. oil	Water/misc. oil
Formula Characteristics:							
Nucleic Acid Incorporation (%)	86 ± 8	67 ± 1.4	50.3 ± 12	39 ± 1.7	33 ± 6	37 ± 1.4	58 ± 16
Low MW DNA Appearance (% above bkground, Post nanocapsule digest by electrophoresis)	15.00	76	93.00	53.00	66	28	41.00
Supercoil retention (post 100 hrs. release) (area %, initial distribution = 76% supercoiled)	87%	65%	66%	59%	43%	65%	80%
Particle Size,(mean ± SE)	42 ± 2	45 ± 3	73 ± 4	226 ± 11	291 ± 25	150 ± 7	199 ± 11
Secondary Structure(s)	25% 100 - 200 nm	30% 500 nm	70% 300 nm	S<10%	S<10%	S>40% yeast-like	S>80% 400 nm
Flocculation Status	stringy flocs	stringy flocs	spheroid aggregates			aggregates	aggregates

Comments:				ppt. during aerosolization		ppt. during aerosolization	ppt. during aerosolization
Performance: Transduced GFP Protein Generation (pixel units, % of control liposome formula, 100 μg total protein, Day 11)	420	340	0	0	0	0	0

Nanocapsule sizing was determined by tapping mode AFM and images are illustrated in FIG. 2A. The data indicate average nanocapsule sizes less than 50 nm are achievable only with multi-phase systems in combination with low water solubility surfactants (Table 1A: Formulae Q,R vs. S,T,U,V, and W). Furthermore, only nanocapsules of less than 50 nm resulted in detectable transgene production in CRL-1764 rat fibroblast cells (Table 1A). Effective dispersion also corresponded with decreased aggregation and enhanced DNA stability (as indicated by decreased electrophoretic breakdown products). The starting DNA was partially relaxed (76% supercoiled by electrophoresis). Using this value as a basis, supercoil retention in DNA still encapsulated following 100 hours of release testing, was excellent in multi-phase systems.

Release profiles for hydrophillic dispersed atomized nanocapsules were linear, showed no zero burst and resulted in about 60% release after 72 hours (See Figure 1B). Formula W, manufactured with a standard surfactant (Tween 80) at a reasonable loading value (0.4%) failed to completely release loaded DNA. Figure 2C illustrates that small amounts of DNA (in this case 300 nanograms of DNA) can be recovered accurately in a procedure comprising butanol extraction of 10% butanol/saline releasing fluid followed by isolation on a miniprep column and measurement of absorbance at 260 nm excitation. Results obtained from UV spectroscopy are confirmed by electrophoresis of recovered DNA following alcohol coprecipitation with a commercial coprecipitant aid. Experiment 1A demonstrates the importance of a multi-phase system in creating coated particles from the micellar solution, defines surfactant requirements and validates method for measuring *in vitro* release profiles.

Example 1B - Effect of process parameters on particle functionality

To investigate the effect of modulating process parameters on nanocapsule functionality for DNA delivery, a series of formulas (designed to release in 3 days) were prepared. The transduction efficiency of these formulas for delivering a nuclear Green Fluorescent Protein (GFP) reporter transgene in rat fibroblast cultures was measured 5 days later. Charge neutralization of the DNA molecule, the surfactant / oil system, total surfactant phase volume, the inclusion of probe sonication, the absolute requirement for atomization and receiving bath osmolality were modulated. Results for this experiment are summarized in the Table 1B:

Table 1B : Effect of process parameters on particle functionality

Nanocapsule Design	*Formula Name*	*charge neutral-ization by con-densor*	*Surfactant*	*Biocomp-atible Oil*	*Oil Phase Volume (%, 4.5 ml basis)*	*Emulsify by sonication*	*Atomize Dia-meter (μm)*	*Receiving bath Osmol-ality (mOs)*
1	q.co.2	+	Cetyl OH	Castor oil/Etoh	4	+	250	220
2	q.co	- -	Cetyl OH	Castor oil/Etoh	4	+	250	220
3	o.35	+	TM-diol	DMSO	4	+	1.4	220
4	ea0.2	+	TM-diol	DMSO	4	- -	- -	220
5	ea0.1	- -	TM-diol	DMSO	4	- -	- -	220
6	ed0.2	+	TM-diol	DMSO	0.05	- -	250	220
7	ed0a.12.di	+	TM-diol	DMSO	0.05	- -	250	0

Nanocapsule Design	*Formula name*	*Nanocapsule diameter(nm)* n=20*	*Encapsulation yield (%, mean ± SE)*	*Transduction Efficiency, (5 days, rat fibroblasts)*
1	q.co.2	20 ± 3, rods	48.6 ± 11	87 ± 7%
2	q.co	12 ± 0.7, irregular	48.6 ± 2	71 ± 28%
3	o.35	17 ± 1.2, spheres	82.3 ± 7 (4)	86 ± 2%
4	ea0.2	24 ± 2, s/r	32 ± 10	72 ± 2%
5	ea0.1	36 ± 3, irregular	57 ± 2	85 ± 1%
6	ed0.2	39 ± 3, r/e	39 ± 5	96%
7	ed0a12.di	39 ± 3, ellipse	69 ± 2	100%

*Nanocapsule diameter is reported as average of the minor and major particle axis using digital image analysis, while nanocapsule morphology is reported as irregular, rods (r), ellipse (e) or spheres (s). As the radius of curvature for the AFM silicon cantilever can be 10-30 nm, dilation effects can result in diameter overestimates by as much as 50%.

Aqueous dispersions of DNA condensates with poorly soluble surfactants in the inventive method produced average nanocapsule diameters under 50 nm. A number of successful operating regimes were feasible with varying effects on encapsulation yield. In a cetyl alcohol/castor oil system, under condensation resulted in an average particle diameter decrease from 20 to 12 nm (Table 1B: F1 vs. F2). The same decrease in condenser weight ratio induced an average particle size increase from 24 to 36 nm, while still maintaining nanocapsule functionality for trangene delivery, when using a TM-diol/DMSO surfactant system for initial micelle formation (Table 1B: F4 vs. F5). This finding teaches surfactant selection impacts final average nanocapsule diameters.

The removal of moderate energy input (dropped probe sonication, atomization but kept bath sonication) during nanocapsule formation resulted in functional particles with decreased yield (Table 1B: F3 vs. F4). This finding indicates that optimal nanocapsule production is not dependent on any spontaneous micro-emulsification process. Cosolvent phase volume was reduced from 4 weight percent to 500 ppm without any negative effect on particle functionality (Table 1B: F4 vs. F6). This finding indicates that essentially solvent-free nanocapsules can be made by the inventive method. Finally, salt was removed from the atomization receiving bath without any negative effects on nanocapsule functionality (Table 1B: F6 vs. F7).

Example 2 - Effect of nanocapsule sizing on a nanocapsule uptake in human keratinocytes

The effect of nanocapsule sizing on intracellular trafficking in immortalized HacaT human keratinocyte cultures (HacaT's) was investigated in this example. In this series of formulae, the micellar dispersion was sheared by syringes of different orifice diameter. The coating weight was also lowered from 1:1 DNA: Polymer (w/w) to 1:40 to shorten the dissolution profile from 5 to 3 days. In these experiments, nanocapsule formulae were compared to standard polyplexes of DNA and PEI, and lipoplexed plasmid DNA. Table 2 summarizes the experimental design and results:

Table 2: Effect of particle size on nanocapsule functionality for gene transfer

Formula Name	*Particle Size (mean, nm; morphology)*	*4 hr. coloca-lization with caveolin-1**	*4 hr. coloca-lization with ~~cathrin~~ clathrin*	*10 hr. coloca-lization with lysosomes*	*Transduction Efficiency, (5 days, human keratinocytes)*
o.22 (64)	47 ± 3, rods	0	++	+	16 ± 13
o.27 (57)	21 ± 2, rods	+	±+	ND	81 ± 8
o.35 (85)	17 ± 1.2, spheres	+++	0	0	78 ± 9
pei-pDNA	67 ± 4, spheres,irreg.	0	+++	+++	40 ± 15
Lipoplex pDNA	48 ± 2 200 nm aggregates	+	+	+++	41 ± 27

Key: 0 = no change from unstimulated condition, + greater than 25% increase, ++ greater than 50% increase, +++ greater than 75% increase in number of cells stimulated. ND = not determined.

It was observed that compared to the unstimulated state, nanocapsules increased cellular pinocytotic activity relative to standard formulations, and smaller nanocapsules shifted pinocytotic activity to caveolae from clathrin-coated pits (Table 2: Formula O vs. pei-DNA and lipoplex pDNA). It was further observed that nanocapsules avoided lysosome co-localization at 10 hours post-addition with smaller nanocapsules being particularly effective (see Table 2: Formula O vs. pei-DNA and lipoplex pDNA). These results are illustrated further in Figure 3. This improvement is further emphasized by comparison with published uptake studies for HacaT keratinocytes. Compared to primary keratinocytes, uptake of naked DNA oligonucleotides (20 μM) was very poor in HacaT's and showed accumulation of oligonucleotides in punctate vesicles consistent with lysosomes at 2 hours. In contrast, using hydrophillic dispersed atomized nanocapsules of the inventive method, complete avoidance of lysosomes at 10 hours post-addition was demonstrated (FIG. 3). These results indicate that products of the inventive process will have increased and prolonged effectiveness.

Example 3 - Effect of nanocapsule delivery on DNA and reagent-induced cytotoxicity.

To test whether soluble exogenous DNA released from liposomes or dendrimers induces apoptosis, Rt-1's were treated with loaded/unloaded liposome complexes, dendrimer complexes, nanocapsule and1 μg/ml etoposide, a DNA intercalating agent as a positive control. Cultures were treated with standard formulas for 3 hours then assayed for gene product expression 30 hours later. Cultures were treated with nanocapsules for 4 days to ensure full DNA release during the experiment. Controls included as a positive control for apoptotic cell death, 1 μg/ml etoposide, a DNA intercalating agent that was applied to cultures overnight before experiment termination. Other controls included standard PEI-DNA complexes, empty nanocapsules and nanocapsules containing empty vector plasmid DNA. Hydrophillic nanocapsules were produced for this experiment as described earlier using a 35-gage syringe.

One of the later steps in apoptosis is DNA fragmentation mediated by activation of endonucleases as part of the apoptotic program. Therefore, DNA fragmentation was assayed by end-labeling of fragments using an exogenous enzyme and a substituted nucleotide (TUNEL: tdt-mediated uridine nucleotide and labeling. Results are expressed as a Fragmentation Index, or the percent of cells in the total culture exhibiting BRDU end-labeled DNA. Cultures were run in duplicate. The experimental design and results are summarized in Table 3:

Table 3: Effect of nanocapsule coating weight on nonspecific reagent and plasmid DNA-associated cytoxicity.

Formula	*κ.35*	*ξ*	*O (Omicron)*	*b.35*	*Y.35*	*Lipoplex GP*	*Lipoplex L+*	*Polyplex*
Particle Design: DNA Condensing Agent	Denatured h. keratinocyte nuclear protein	100Kd MW Polylsine	27 kD PEI	27 kD PEI	27 kD PEI	cationic lipid	cationic lipid	dendrimer
Coating Ratio (DNA/polymer)	0.1	0.25	0.25	0.01	0.0025			
Performance: Dose: (30 hrs for Std. Formulas, 100 hrs for nanocapsules)	4.6	4.1	4	5	5	1 μg 500 ng 0 ng	500 ng 250 ng 0 ng	2 μg 1 μg 0 μg
Cytotoxicity: (Fragmentation Index, %)	ND	0.26 ± 0.15	2 ± 0.7	1.9 ± 0.6	9 ± 8	27 ± 8 6 ± 3 4 ± 2.5	9.3 ± 0.2 12.8 ± 1.5 7.8 ± 0.1	6.63 ± 1.4 5.7 ± 1.8 3.1 ± 0.3
Cytotoxicity controls: (1 μg etoposide (8 hr): 25 ± 10%) (Pei-DNA polyplexes (100 hr): 24 ± 7%) (Empty vector nanocapsules: 1.25 ± 1.25%) (Empty vector nanocapsules:	31 ± 2	ND	85 ± 7	32 ± 3	24 ± 4	17 ± 2	dead	dead

0.9 ± 0.7%) Transduction Efficiency: (% cells) 120 hrs, dose as listed)								
Formula Characteristics:								
Nucleic: Acid	55 ± 10	27 ± 7	54 ± 5	65 ± 4	667 ± 0.2	ND	ND	ND
Incorporation: (%)	70	75 ± 8	83 ± 12	ND	ND	ND	ND	ND
Cumulative Release: (%, 48hr)	26 ± 2	22 ± 2	20 ± 1	35 ± 2	57 ± 5	48 ± 2	ND	22.4 ±2
Particle Size (mean ± SE, nm) Agglomerates (as dispensed)	few	50% 80±6	200 nm	200 nm	g.t. 50% 300 nm	300 nm		25% 300 nm hard-fused

Table 3B: Dose response of nanoencapsulated pDNA

Formula	Dose (100 hr.)	GFP/Action Production (density ratio, %)
K.35	9 µg	94.8
K.35	4.5 µg	83.5
K.35	1.5 µg	83.3
Lipoplex GP	0.5 µg	94.9

It was observed that use of controlled-release nanocapsules reduced the fraction of apoptotic cells in fibroblast cultures 3 to 100 fold. Conventional reagents without DNA showed a 4-fold increase in FI (Fragmentation Index) over empty nanocapsules, but increased another 50-100% in the presence of additional DNA without additional reagents. Decreasing the coating weight from 1:40 to 1:400 resulted in an increase in average nanocapsule diameter from 20 to 57 nm and the appearance of regions of apoptotic induction in cultures (Table 3: Formula omicron vs. Formula upsilon 35). Decreasing the coating weight from 1:40 to an intermediate 1:100 reduced transduction efficiency without increasing particle size and the appearance of cytotoxicity. These findings indicate that nanocapsule design plays a role in maintaining nanocapsule integrity and that size effects and dissolution profiles can contribute to observed cytotoxicity and functionality. We concluded that application of nanocapsule formulations increased dosing to useful efficiency levels without induction of an apoptotic program.

Table 3B exemplifies this improvement with a dose response of Formula K.35 measured in fibroblast lysates. GFP production was measured in fibroblast lysates after 4 days of treatment with increasing doses of nanocapsules. A 9.5 μg dose of nanocapsules equaled the production of a liposomal formulation without any evidence of cytotoxicity.

Example 4 - Nanocapsule delivery of macromolecules to porcine tissue across keratinized barrier epithelia by transdermal and subcutaneous means.

The utility of nanocapsules for nonviral nucleic acid delivery to tissue in a pig biopsy organ culture system was investigated. 6 and 8 mm circular biopsies were collected under sterile conditions from sedated research animals and cultured on meshes in partial contact with media containing 20% Fetal Calf Serum. Biopsies were either injected with 90 μl (6.3 μg) or treated topically with 3 x 30 μl aliquots. Biopsies were snapfrozen 7 days later and sectioned/homogenized for beta-galactosidase production measurement. Formulation information and results from this experiment are summarized in Table 4:

Table 4: Functionality of dispersed atomized nanocapsules for macromolecule delivery across keratinized barrier membranes.

Formula	N	O
Exp. Modification (from Formula Q)	coating wt. is 2.5x Polymer MW is 1x	coating wt. is 2.5x Polymer MW is 4x
Formula Characteristics: Nucleic Acid Incorporation (%) Cumulative Release (%, 169 hr. 2.5 µg sample) Low MW DNA in postdigested Electrophoresis Samples Supercoil retention (237 hr. release, initial=69.7% **sc**/relaxed) Particle Size (mean ± SE, major species) Particle Description Secondary structure: **Performance:** Transduced Protein Production (pixel units, % of neg. control, 100 µg total protein, normalized by protein)	 70.00 83 0 100% 18.2 ± 0.2 nm spherical 20% 100 nm yeast-like clusters 312 ± 74 (topical) 142 (s.c.)	 70.50 83.5 ± 1.5 0 100% ND 20% 100nm yeast-like clusters
Reporter Gene Product Distribution (6.3 µg dose, 6 mm (N), 8 mm (O) porcine biopsy, 1 **wk**.) keratinocytes (% cells), n = 2 fields/200 cells, negative control: 6% endothelial cells, (% vwf- + area) papillary and/or reticular, n = 2 - 4 fields, negative control: 1.07 ± 0.72 dermis, (% area); negative control: 0.24 ± 0.03, n = 4/20x fields	 100% 73 ± 20 (papillary) * 32 ± 15 (reticular) 2.74 ± 0.96 *	 100% 13.8 ± 0.5 (papillary) * 8 ± 2 (reticular) * 1.77 ± 0.49 *

* = $p < 0.05$

Western blotting of radiated tissue lysates showed a 3-fold increase in beta-galactosidase in duplicate biopsies treated topically with Formula N over an identically cultured 6mm biopsy treated with saline. Only a 2-fold increase was measured in a 8 mm biopsy treated topically with formula O nanocapsules (see FIG. 5A). Formula O was produced with a higher molecular weight analog of the Formula N polymer suggesting a difference in particle

morphology, a dose effect or differing *in situ* release profiles between the two formulations related to this difference. To identify initial cell type-specific differences in nanocapsule delivery effectiveness, tissue sections were analyzed for beta-galactosidase expression in double-label experiments using antibodies to cell-specific epitopes (see FIG. 5B). Digital image analysis of these sections indicated that radiated keratinocytes and endothelial cells are readily transduced in organ culture 7 days after treatment with a 10 day releasing formula. Specific quantitation of fibroblastic cells was not possible without inclusion of a cell-specific marker, however, an 11-fold increase in area of expression was measured in N biopsy dermis (see Figure 4B). Interestingly, for both the formulae N and O topically-treated biopsies examined, the area percentage of blood vessels transduced decreased about 50% in nearby fields between 100 μm and 300 μm of depth (Table 4: papillary (pap) vs. reticular endothelial (ret) cells). These data suggest that nanocapsules are penetrating the epidermis to enter the dermis.

Example 5- Incorporation of inventive nanocapsules into a solid dosage form for additional utility in physical targeting.

Nanocapsules containing a nuclear GFP transgene or empty vector were incorporated into a suture coating by vortexing the following components: i) 50 μg of nanocapsules containing plasmid DNA, ii) 200 μg of bovine mucin, and iii) 75 μg of sucrose (60% w/w) in a 1000 μl volume. Sutures were aseptically coated by drawing sutures 5x through punctured microcentrifuge tubes containing the coating. Coating functionality for gene transfer was tested by applying sutures in cultured porcine skin biopsies. Biopsies were cultured on a mesh such that the biopsy bottom was in contact with cell culture media. Biopsies were treated for 7 days, then snap-frozen and sectioned for immunofluorescence microscopy to assess nanocapsule penetration and transgene delivery.

Nanocapsule penetration was detected by streptavidin-biotin immunocomplexes directed at sheep IgG. Nanocapsule coatings are spiked with ovine IgG to enable this detection strategy. Figure 6A shows distribution of sheep IgG signal throughout porcine dermal tissue with accumulation on capillaries. In Fig 6A', primary antibody is omitted during slide processing to determine level of background fluorescence. A suture is visible in this view. Sutures were identifiable as smooth objects without positive nuclear counterstain. GFP expression was confirmed using a polyclonal GFP antibody to obviate the effect of nonspecific tissue green fluorescence. Figure 6B shows nuclear-localized GFP expression throughout the suture-treated dermis using a GFP polyclonal antibody. A suture was visible 750 microns away. Figure 6C shows the lack of GFP expression in a biopsy treated with empty vector coating. This example demonstrates the usefulness of nanocapsules for use in physically targeted macromolecule delivery.

Example 6- Utility of nanocapsules for local targeting by design of nanocapsule coating.

Fibroblast targeting

GFP nanocapsules were prepared by dispersion atomization as described in Example 1. Polyvinylpyrylodone (PVP, MW 10,000) was used as the coating basis. A coating weight ratio of 1:40 was used and rod-shaped nanocapsules of 23 ± 2 nm were produced. 1 μg of PVP nanocapsules were applied to both human dermal fibroblasts (HDF) and HacaT keratinocyte cultures for 4 hours then fixed for detection for nanocapsule uptake by streptavidin-bioting immunocomplexes to sheep IgG. Nanocapsule coatings were spiked with ovine IgG to enable this detection strategy. Figure 7A illustrates positive nuclear localization of PVP nanocapsules in HDF's and negative colocalization of PVP nanocapsules in keratinocytes (Figure 7A: 7Aa vs. 7Ab). Views of untreated cultures are included for comparison (7Aa', 7Ab,). Cultures were also treated with 5 μg of PVP nanocapsules for 5 days then tested for GFP transgene production. Consistent with uptake studies results, only the fibroblast cultures showed production of GFP transgene (7A: 7Aa'' vs. 7Ab'').

Tumor-targeting

GFP nanocapsules were prepared by dispersion atomization as described in example 1. Tenascin (TN, MW 200,000) was used as the coating basis. A coating weight ratio of 1:20 was used and spherical nanocapsules of 19 ± 0.9 nm were produced. 500 ng of TN nanocapsules were applied topically in successive small aliquots to pig biopsies maintained in organ culture. Biopsies were rinsed in media after 3 minutes of topical application and followed by exchange for new media in the organ culture to preclude any delivery other than topical.

To simulate tumor nests of epithelial-derived origin, biopsies had been seeded 12 hours previously with 50,000 human squamous carcinoma cells. 7 days later biopsies were snapfrozen and sectioned for immunological detection of GFP production. In Figure 7B, view "a" shows intense GFP fluorescence in the tumor center, view "b" confirms this GFP expression with polyclonal antibodies to GFP, view "c" shows cell positioning in the section using a counterstain for cell nuclei and view "d" shows the level of background fluorescence by omission of GFP antibodies. Tumor origin was confirmed by positive detection with antibody to keratin 10/1, an epithelial marker. Comparison of view "b" and view "c" indicates that GFP expression is limited to cells within the tumor. As already demonstrated in example 5, expression throughout a tissue is also feasible and can modulated by coating design. This example demonstrates that nanocapsule delivery can be productively targeted.

Cell-specific delivery for enhanced drug therapeutic window

Nanocapsules were prepared as described in Example 1 to encapsulate cisplatin, a hydrophobic molecule and a common cancer chemotherapeutic with serious side effects. A

coating weight ratio of 1:100 was used and irregular nanocapsules of 29± 3 nm were produced. Targeting efficacy was demonstrated by changes in the dose response for cell growth inhibition in fibroblast vs. squamous cell carcinoma cultures. Cells were seeded overnight into 96 well plates, treated for 18 hours with increasing amounts of encapsulated or naked drug. Drug was then removed and cultures were assessed for cell growth inhibition using an MTT assay 48 hours later for a total growth time of 72 hours. Results are illustrated in Figure 7C. The data shows that tenascin nanocapsules protected non-target cells from cell death (zero death) at drug levels that killed non-target cells using naked drug (Figure 7Ca: open vs. closed circles). In carcinoma cultures, TN nanocapsules productively decreased the inhibition concentration (IC50) for cell survival an estimated 200% from 350 to 165 μg/ml. Example 6 demonstrates the usefulness of nanocapsules for use in coating- targeted macromolecule delivery.

Example 7 -Utility of nanoencapsulation for improved cellular uptake of other species used as pharmaceutical, nutraceutical, research or cosmetic agents.

Nanocapsules containing either 500 kD Fitc-labeled dextran, 20 mer Fitc-labeled mer O-methylated RNA oligonucleotide and 16 mer phosphodiester DNA oligonucleotide were prepared as described in Example 1. A 1:40 coating weight ratio was used and 1 MM kD recombinant hyaluronan was used a coating basis. PEI was used to condense the phosphodiester DNA oligonucleotide, but no PEI was included in the dextran or RNA oligonucleotide nancapsule formulas. Nanocapsule functionality for drug delivery was tested by evaluating changes in cellular uptake and lysosomal activity in 35 mm cultures of human dermal fibroblast. Nanocapsule formulas were compared to naked species or species formulated as complexes. Quantitative results are summarized in Table 7.

Table 6. Nanoencapsulation improves cellular uptake of other species used as pharmaceutical, nutraceutical, research or cosmetic agents. At 18 hours post-addition, lysosomes are only evident in conventionally formulated species.

			4.5 hours post-addition		18 hours post-addition	
Bioactive Component	Formulation	Particle size (mean, SE, nm, morphology)	Increase in cellular uptake activity, (% cells above baseline, caveolin-1 /clathrin) dose	Nuclear Uptake Efficiency (% cells, fibroblast)	Bioactive component Colocalization with lysosomes, (% cells, human fibroblasts) dose	Detection persistence, (% cells, human fibroblasts)
500 kd fitc-dextran	nanocapsule	22 ± 2, s/r	89 / 20 25µg*	95 ± 2	2 ± 2 5 µg	88 ± 11
	naked, Fitc-labelled	- -	75 / 18 100 µg	10	100 ± 10 100 µg	61 ± 20
20 mer o-methylated RNA oligo	nanocapsule	13 ± 0.7, r	78 / 90 2 µg	74 ± 5	0 ± 0 5 µg	80 ± 6
	naked, Fitc-labelled	- -	- - / 73 5 µg	14 ± 7	- -	- -
	PEI/Fitc-labelled	236 ± 26, r	- - / - - - -	- -	100 ± 0 5 µg	94 ± 10
16 mer PO DNA oligo	nanocapsule	17 ± 1, r	70 / 94 1 µg	34 ± 25	0 ± 0 5 µg	91 ± 8
	PEI/Fitc-labelled	67 ± 4, s/r	72% 2 µg lysosomes	95 ± 2	80 ± 7 5 µg	66
Nominal **n**		20 particles	70 cells	140 cells	50 cells	50 cells

* Dose was estimated for encapsulated dextran assuming 100% encapsulation.

s = sphere

r = rod

Table 7 shows that average diameters for all nanocapsules were below 50 nm byAFM. PEI complexes of DNA oligonucleotides were measured at 67 nm and PEI complexes of uncharged RNA O-methyl oligonucleotides were measured at 236 nm. As discussed in Example 2 using keratinocyte cultures and plasmid DNA, nanocapsules stimulate uptake activity as indicated by increased signal levels of clathrin and caveolin-1. In the 500 kD dextran case, uptake activity shifts productively towards caveolae and potocytosis with nanoencapsulation (Table 7, 500 kD Dextran). At 4.5 hours post-addition, nuclear uptake is enhanced for encapsulated dextran and RNA relative to naked species.

For the case of DNA oligonucleotides, cellular uptake of the nanoencapsulated oligonucleotides is decreased relative to complexed oligonucelotide. However, by 4.5 hours post-addition, a majority of the simply complexed DNA oligonucleotides is already nonproductively sequestered in lysosomes (Table 7). At 18 hours post-addition, nanocapsule species show continued exclusion from lysosomes, while the DNA oligonucleotide polyplexes show high levels of sequestration.

This pattern of nanocapsule exclusion from lysosomes and polyplex sequestration in lysosomes is illustrated in Figure 8A and 8B for an O-methyl RNA oligonucleotide species labeled with fluorescein (FITC). Views 8Aa and 8Ba show Fluorescein detection in cultures at 18 hours post-addition indicating that distribution is exclusively nuclear for the nanoencapsules of RNA oligonucelotides (Figure 8B: a vs. a'). Punctate inclusions are visible that co-localize with an immunological marker for lysosomes in the cultures treated with RNA oligonucleotide polyplexes (Figure 8A:a vs a'). Particle sizing results from AFM microscopy for polyplexes and nancapsules are included to demonstrate the dramatic differences in sizing following nanoencapsulation. (Figure 8A, 8B:8Ab vs. 8Bb, 8Bb'). Formulas encapsulating lower molecular weight dextrans and unstabilized RNA were also prepared with similar uptake, nanocapsule size and yield to demonstrate that nanoencapsulation can provide not only a targeting function but aid in stabilizing molecules sensitive to chemical or enzymatic degradation. These examples demonstrate the usefulness of nanocapsules 36 for use in delivery of a broad range of macromolecules.

Although the present invention has been described with reference to preferred embodiments, workers skilled in the art will recognize that changes may be made in form and detail without departing from the spirit and scope of the invention.